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# Genome-wide gene expression profiling suggests distinct radiation susceptibilities in sporadic and post-Chernobyl papillary thyroid cancers

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Papillary thyroid cancers (PTCs) incidence dramatically increased in the vicinity of Chernobyl. The cancer-initiating role of radiation elsewhere is debated. Therefore, we searched for a signature distinguishing radio-induced from sporadic cancers. Using microarrays, we compared the expression profiles of PTCs from the Chernobyl Tissue Bank (CTB,  $n = 12$ ) and from French patients with no history of exposure to ionising radiations ( $n = 14$ ). We also compared the transcriptional responses of human lymphocytes to the presumed aetiological agents initiating these tumours,  $\gamma$ -radiation and  $H_2O_2$ . On a global scale, the transcriptomes of CTB and French tumours are indistinguishable, and the transcriptional responses to  $\gamma$ -radiation and  $H_2O_2$  are similar. On a finer scale, a 118 genes signature discriminated the  $\gamma$ -radiation and  $H_2O_2$  responses. This signature could be used to classify the tumours as CTB or French with an error of 15–27%. Similar results were obtained with an independent signature of 13 genes involved in homologous recombination. Although sporadic and radio-induced PTCs represent the same disease, they are distinguishable with molecular signatures reflecting specific responses to  $\gamma$ -radiation and  $H_2O_2$ . These signatures in PTCs could reflect the susceptibility profiles of the patients, suggesting the feasibility of a radiation susceptibility test.

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An increased incidence of thyroid carcinomas in children was first noticed in Belarus and Ukraine 4 years after the 1986 Chernobyl accident (Baverstock *et al*, 1992; Kazakov *et al*, 1992). Increased incidence has been observed since then in people exposed to fallout during childhood in these regions (United Nations Scientific Committee of the Effect of Atomic Radiation, 2000; Mahoney *et al*, 2004). The aggressiveness and morphology of these tumours (over 95% classified on the basis of their pathology as papillary thyroid carcinomas (PTCs)) appear to be related to the age of the patients at the time of the accident and to the lag between the accident and diagnosis, that is, the latency of the cancers (Williams *et al*, 2004).

Radiation is the only proven cause of PTC so far. Although the cause of PTC in patients not exposed to radiation remains uncertain, a growing body of evidence suggests that  $H_2O_2$  could play a role in the absence of radiation. Indeed, it is a potent DNA-damaging agent produced in large amounts during thyroid hormone synthesis (Corvilain *et al*, 2000). It causes DNA damage (guanine oxidation, single- and double-strand breaks) in human

lymphocytes (Turner *et al*, 2003), hamster ovarian cells (Dahm-Daphi *et al*, 2000; Mondello *et al*, 2002), and in human, dog and sheep thyroid cells in primary culture (Chico Galdo *et al*, 2006). Hydrogen peroxide is believed to destroy follicular thyroid cells in myxoedematous endemic cretinism (Kohrle *et al*, 2005) and to cause cancers in the thyroid of Tg- $\alpha_{1B}$ AR mice (Ledent *et al*, 1997). Lack of protective systems, peroxiredoxin or glutathione peroxidases, in knockout mice lead to cancer (Neumann *et al*, 2003; Lee *et al*, 2006). Transfection of an  $H_2O_2$ -generating system transform epithelial cells (Chu *et al*, 1996). The spontaneous somatic mutation rate in normal mice and rat thyroid cells is substantially higher than in liver and lung cells (Corvilain *et al*, 1994). With a turnover of 8.5 years in adults (Coclet *et al*, 1989), thyrocytes have time to accumulate  $H_2O_2$ -induced DNA damages. Hydrogen peroxide has been found to play a role in several human cancers (Quinn *et al*, 2006). Thus, a number of arguments support a role of  $H_2O_2$  in the initiation of PTC, and in particular in patients not exposed to radiation.

The vast majority of PTCs harbour either a BRAF mutation (45%; Xing, 2005) or a RET/PTC rearrangement (35% in adults; Nikiforov, 2002), which are generally mutually exclusive (Soares *et al*, 2003). Both gene alterations result in the constitutive activation of the RAS–RAF–MAPK signalling pathway (Kimura *et al*, 2003; Soares *et al*, 2003). Gene-expression signatures separating BRAF from RET/PTC tumours have been reported,

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but the number of genes involved varies from a few dozens (Frattoni *et al*, 2004) to several thousands (Giordano *et al*, 2005). Although early reports pointed at a lower BRAF mutation frequency in Chernobyl patients, recent evidence suggests that the BRAF mutation is associated with age and is more prevalent among older Chernobyl patients and/or among patients with longer latency tumours (Kumagai *et al*, 2004; Lima *et al*, 2004; Powell *et al*, 2005; Rosenbaum *et al*, 2005). Several research teams have reported higher frequencies of RET/PTC rearrangements in post-Chernobyl patients (Nikiforov *et al*, 1997). These higher frequencies could result from the fact that radiation induces double-strand breaks, and thus rearrangements rather than point mutations (Dahm-Daphi *et al*, 2000), or possibly to a differing molecular profile in childhood vs adult papillary carcinomas (Powell *et al*, 2005). The induction of RET/PTC rearrangements after *in vitro* irradiation of immortalised thyroid cells (Caudill *et al*, 2005) supports the former explanation. Whether the two best-characterised genetic alterations found in PTC are involved in a radiation signature remains an open question. In addition, radiation induces other unknown alterations.

In this paper, we have compared the gene-expression profiles of PTCs from adult French patients with no history of exposure to radiation and from adult Ukrainian patients exposed to Chernobyl fallout during childhood, and asked whether there is a gene-expression signature distinguishing radiation-induced from sporadic cancers. Our preliminary investigation suggested the absence of a large-scale radiation signature (Detours *et al*, 2005). We extend it here by using a more recent microarray technology, by covering more genes, by studying more patients and by establishing results with a wider range of statistical methods. We confirm that French and Chernobyl Tissue Bank (CTB) tumours have the same overall expression profiles and have indistinguishable BRAF and RET/PTC frequencies.

We also compared the transcriptional responses of human cells to the two most likely aetiological agents of CTB and French tumours;  $\gamma$ -radiation and  $\text{H}_2\text{O}_2$ . The similarity of CTB and French tumours is mirrored by the similarity of the transcriptional responses to  $\gamma$ -radiation and  $\text{H}_2\text{O}_2$ . However, subtle expression differences are exploitable to accurately classify these tumours according to their origin. Part of these expression differences includes genes involved in the differential response to  $\text{H}_2\text{O}_2$  and radiation, and genes involved in homologous recombination which suggests that different—and detectable—susceptibility profiles lead to sporadic and radiation—induced PTC.

## MATERIALS AND METHODS

### Transcriptional and genetic data

Paired samples of tumoral and adjacent non-tumoral thyroid tissues were obtained from the CTB ([www.chernobyltissuebank.com](http://www.chernobyltissuebank.com)) and from patients undergoing surgery for thyroid disease at the Ambroise Pare Hospital (Boulogne, France). French tissues were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. Diagnoses were made by the Department of Pathology at the Ambroise Pare Hospital or by the International Pathology Panel of the CTB. The protocol received approval from the Ethics Committees of the institutions. The detail of BRAF-RET/PTC status determination, RNA processing and microarray data preprocessing is available in Supplementary information file S3. Microarray data are available from the Gene Expression Omnibus ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)), accession number GSE3950.

### Comparison of microarray platforms

Jarzab *et al* (2005) data were downloaded from [www.genomika.pl/thyroidcancer/PTCCancerRes.html](http://www.genomika.pl/thyroidcancer/PTCCancerRes.html). We used the original MAS 5.0

normalised expression levels, took the  $\log_2$  of expression ratios and averaged over patients. The probes of the two platforms could be matched on the basis of their Entrez IDs for 4203 genes.

### Unsupervised classification

Hierarchical clustering was computed with the R language function `hclust` with Ward linkage. Multidimensional scaling was computed with the R function `isoMDS`. Both methods were fed Pearson correlation distances as input.

### Supervised classification

Support vector machine classification was run with linear kernel and  $\text{cost} = 1$  using the `rfe` 0.2 and `e1071` 1.5.9 packages for R. The generalised partial least-square (GPLS) implementation from package `gpls` 1.1.0 (Ding and Gentleman, 2004) for R was run with default parameters. Prediction analysis of microarray (Tibshirani *et al*, 2002) was run with threshold values in  $\{1.0, 1.1, 1.2, \dots, 3.0\}$  using `pamr` 1.25 for R. The random forest classification used default parameters from R package `randomForest` 4.5.12 (Zhang *et al*, 2003). Generalised partial least-square and random forest (RF) were combined with an external genes selection procedure focusing on the  $n$  genes with the highest absolute  $t$ -statistics, with  $n$  in  $\{1, 2^1, 2^2, \dots, 2^{13}\}$ . We adopted the inner/outer cross-validation scheme described in details in Ruschhaupt *et al* (2004) and implemented in the package `MCReestimate` 1.3.0 to prevent parameter and gene selection biases (Ambroise and McLachlan, 2002). Note that a simpler split-sample validation, in which samples are not recycled as in the current cross-validation protocol, would be suboptimal here because of the limited availability of CTB samples (Simon *et al*, 2003). A 13-fold cross-validation protocol with each round including parameter and gene selection, and classification was run. At each one of the 13 rounds, the best parameters (including signature size) were estimated by running a nested (inner) 12-fold cross-validation for each combination of parameters. Table 2 presents averages over 10 repetitions of the entire inner/outer cross-validation, each based on a different random 13-fold partitions of the data. The random error was computed by averaging the error of five runs of the complete classification procedure on data with CTB and French labels randomly assigned to samples. The same protocol was used for the classification on the basis of the 118 genes  $\gamma$ -radiation vs  $\text{H}_2\text{O}_2$  signature, except that the number of genes,  $n$ , was chosen in  $\{1, 5, 10, \dots, 118\}$  and that the tested prediction analysis of microarray (PAM) thresholds were in  $\{0.1, 0.2, \dots, 3.0\}$ . Classifications on the basis of DNA repair signatures were run without gene selection, and therefore without inner cross-validation. The PAM threshold was set to 0.5. All  $P$ -values were derived by running 1000 times the complete cross-validation with CTB and French labels assigned randomly to samples and counting how many runs produced classification error below the error obtained on the actual data.

### Derivation of the $\gamma$ -radiation vs $\text{H}_2\text{O}_2$ signature

We downloaded the Supplementary data set S2 of Amundson *et al* (2005) from the *Oncogene* web site ([www.nature.com/onc/index.html](http://www.nature.com/onc/index.html)). Genes with expression values differing by 1.5-fold between the 2.5 Gy  $\gamma$ -radiation- and  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ )-treated TK6 cells were selected. To remove immune system-related genes, we downloaded the `gcma`-processed version of the GNF human gene atlas (Su *et al*, 2004; [symatlas.gnf.org](http://symatlas.gnf.org)), which contains expression profiles of normal tissues in most organs. We performed an unpaired two class Significance Analysis of Microarrays (SAM; Tusher *et al*, 2001) with class no. 1 including immune system-related tissues and white blood cells and class no. 2 including all other tissues. We selected the 20% top-ranking genes, which were

all significant at  $q < 0.05$ , and removed them from the  $\gamma$ -radiation and  $H_2O_2$  signature.

# RESULTS

## Expression profiles and gene alteration status of PTCs from France and from the Chernobyl Tissue Bank

Expression profiles were determined for the tumours of 14 patients from France with no documented history of exposure to radiation, and 12 tumours from the Chernobyl Tissue Bank (see online Materials and Methods). CTB tumours are papillary cancers collected in young people who were exposed to the Chernobyl accident ((Thomas *et al*, 2000), see patient information, Table 1). There are 9 tumours of classical subtype, 4 of follicular subtype and 1 of trabecular subtype among the 14 French PTC samples. There are 8 classical, 3 follicular and 1 solid subtypes among the 12 CTB PTC samples. Three French and four CTB mRNA samples (PTC6, PTC7, PTC11 and S405, S420, S422, S423) were reused from our earlier study (Detours *et al*, 2005).

The mRNA expression profiles of all tumours were determined with 12 000 EST (8000 genes) cDNA microarrays using patient-matched nontumoural adjacent tissues as controls. To assess the quality of the data, we compared our expression ratios averaged over samples with those of Jarzab *et al* (2005), who used the Affymetrix® platform. Pearson's correlation measured on the ~4000 genes available and expressed in both platforms was 0.72 (Figure 1).

Tumours were screened for the presence of a RET/PTC rearrangement and for BRAF V600E mutation (Table 1). A RET/PTC rearrangement was found in 42% (5/12) of the CTB tumours and in 21% (3/14) of the French tumours. The difference between the two groups is not significant according to Fisher's exact test. The BRAF mutation is found in comparable proportions in French (36%, 5/14) and CTB tumours (41%, 5/12). None of these alterations was detected in 30% (8/26) of the tumours.

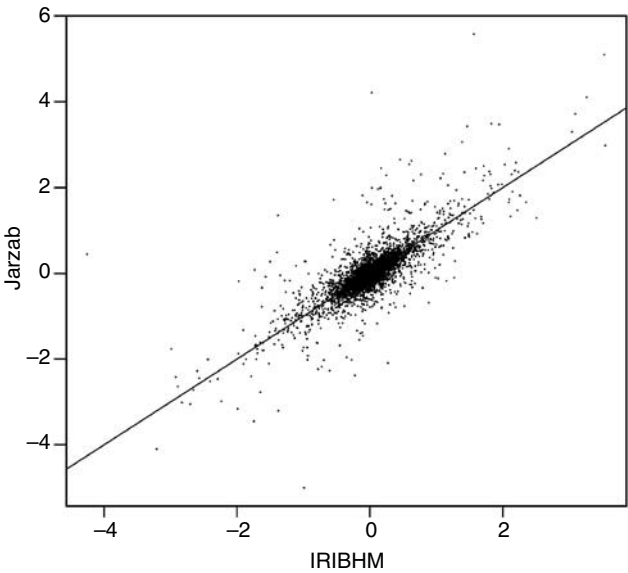
**Table 1** Patient information and gene alterations

| Sample ID | Origin | Sex | Age in 1986 | Age at operation | BRAF | RET/PTC |
|-----------|--------|-----|-------------|------------------|------|---------|
| PTC11     | FR     | F   | 22          | 37               | —    | —       |
| PTC14     | FR     | M   | 17          | 32               | —    | —       |
| PTC18     | FR     | F   | NA          | 59               | +    | —       |
| PTC19     | FR     | M   | 54          | 68               | —    | +       |
| PTC20     | FR     | F   | 54          | 68               | +    | —       |
| PTC21     | FR     | F   | 39          | 54               | —    | +       |
| PTC22     | FR     | F   | 44          | 60               | —    | —       |
| PTC23     | FR     | M   | 17          | 33               | —    | —       |
| PTC25     | FR     | F   | 49          | 60               | —    | —       |
| PTC26     | FR     | F   | 36          | 47               | +    | —       |
| PTC6      | FR     | M   | 24          | 37               | —    | —       |
| PTC7      | FR     | F   | 13          | 29               | +    | —       |
| PTC8      | FR     | M   | 22          | 36               | —    | +       |
| PTC9      | FR     | F   | 24          | 38               | +    | —       |
| S404      | CTB    | F   | 1           | 16               | —    | —       |
| S405      | CTB    | F   | 1           | 16               | —    | +       |
| S409      | CTB    | F   | 11          | 28               | +    | —       |
| S414      | CTB    | F   | 16          | 33               | —    | +       |
| S415      | CTB    | M   | 12          | 28               | +    | —       |
| S418      | CTB    | M   | 10          | 27               | +    | —       |
| S420      | CTB    | F   | 12          | 28               | —    | —       |
| S422      | CTB    | M   | 15          | 31               | +    | —       |
| S423      | CTB    | F   | 5           | 22               | +    | —       |
| S425      | CTB    | M   | 3           | 19               | —    | +       |
| V519      | CTB    | F   | 2           | 18               | —    | +       |
| V608      | CTB    | F   | 15          | 32               | —    | +       |

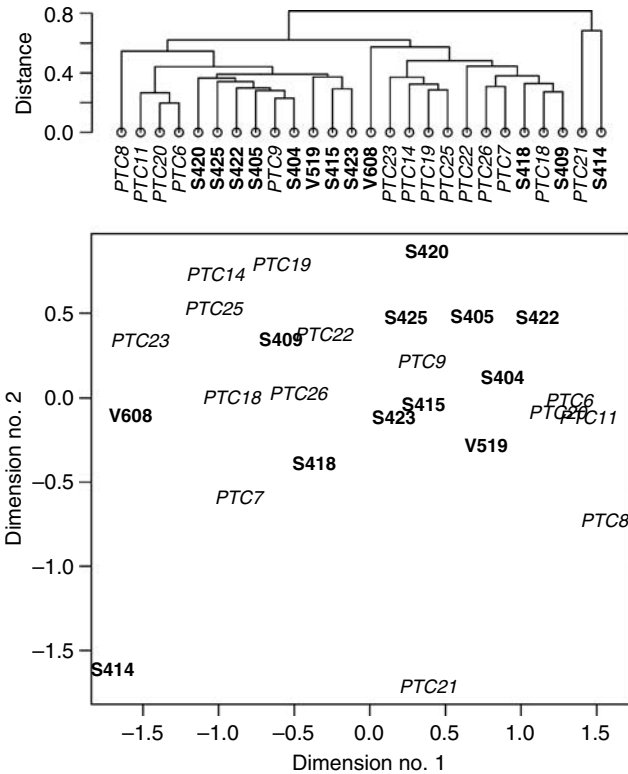
F = female; FR = France; CTB = Chernobyl Tissue Bank; M = male; NA = not available; PTC = papillary thyroid cancer.

## Chernobyl Tissue Bank and French PTCs have similar overall expression profiles

We first searched for global expression differences between CTB and French PTCs, that is, extensive differences detectable when all the genes present on our arrays are considered. Hierarchical



**Figure 1** IRIBHM vs Jarzab *et al.* (2005) microarray data. Pearson correlation between patient-averaged  $\log_2$  tumour/normal ratios of the two studies is 0.72.



**Figure 2** Global expression profiles. Top panel: hierarchical clustering on the basis of all genes. Bottom panel: multidimensional scaling on the basis of all genes. Distances in the two dimensions space were on average distorted by 11% compared to the actual 8000 dimensions gene space distances. Chernobyl Tissue Bank tumours are in bold font, French tumours in italics.

clustering based on all genes did not reveal a clear separation between these two classes of PTCs (Figure 2, upper panel).

Multidimensional scaling collapses the high-dimensional genes space into two dimensions while preserving the distance relationships between all pairs of samples (Figure 2, lower panel). Figure 2 confirms that French and CTB tumours have similar expression profiles when compared on a global scale although CTB tumours form a more compact group.

#### Four supervised classification algorithms find multigenes signatures separating CTB from French PTCs

The absence of separation between CTB and French PTCs on the basis of all genes or at the level of individual genes, does not exclude that these tumours are distinguishable on the basis of a subset of genes. We investigated this possibility with a supervised classification approach (details in Materials and Methods). To strengthen the reliability of our conclusions, all the results were reproduced with four linear classification procedures: linear kernel support vector machines (LKSVM), GPLS, PAMs and RF. Each one included or was combined with a gene selection procedure, that is, a procedure to uncover multigenes signatures including as few genes as possible. All four approaches were tested using a rigorous inner/outer cross-validation procedure (Materials and Methods). It guaranteed that classification testing was performed on independent samples not used for classifier training. The cross-validation results are presented in Table 2A.

The best performer was GPLS. It misclassified 17% of CTB tumours as French PTC, 7% of French PTCs as CTB, resulting in an overall error rate of 12%. Running the same classification on data in which the 'CTB' and 'French' labels were randomly assigned to the 26 tumour samples led to high error rates of 45% ( $n=5$ , s.d. = 12%, see Materials and Methods), as expected for random classification of slightly unbalanced classes (12 CTB and 14 French samples). Thus, the low error rates were unlikely to result from artefacts, including data overfitting. Figure 3 shows the 256 most classifying genes found by GPLS/*t*-statistics trained on all 26 samples (corresponding genes listed in Supplementary Table S1). The optimal signature size varied among the different cross-validation runs from one gene to several thousands of genes, with a median of 256 genes. Such limited stability is widespread,

including in large studies (Ein-Dor *et al*, 2005; Michiels *et al*, 2005). The three other classification procedures, LKSVM/RFE, RF/*t*-test and PAM produced a global error of 15, 23 and 27%, respectively. Thus, undirected selections of classifying genes lead to separation CTB and French tumours.

Note that if the classification results were confined to a subtype, the accuracy would not be as low as 15%, it would be greater than 35%—the classical subtype is the largest, representing 65% of our tumours.

#### Hydrogen peroxide and $\gamma$ -radiation elicit similar transcriptional responses in lymphocytes

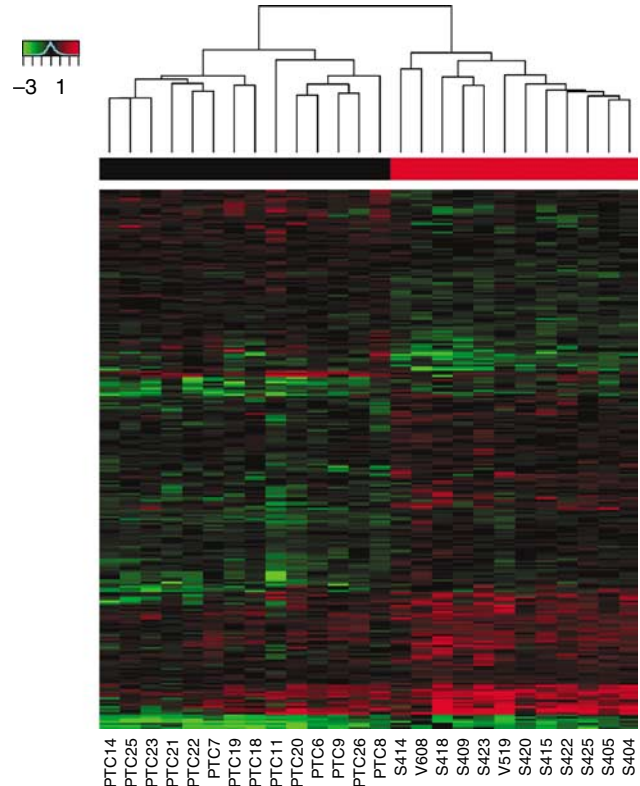
Because hydrogen peroxide,  $H_2O_2$ , is produced at high levels during thyroid hormone synthesis (Corvilain *et al*, 2000) and is a well-known DNA-damaging agent, we investigated the possibility that in the absence of an obvious external risk factor, for example radiation, French cancers must have occurred as a result of  $H_2O_2$  exposure.

Amundson *et al* (2005) measured with microarrays the transcriptional responses of a B-lymphocyte cell line, TK6, to 13 stress agents. These included 10 DNA-damaging agents:  $H_2O_2$ , radiation (neutron and  $\gamma$ -rays at 2.5 and 8 Gy), adriamycin, arsenite, camptothecin,  $CdCl_2$ , cisplatin, methyl methanesulphonate and UVB (280–320 nm). We downloaded the expression data published with the paper and produced the hierarchical clustering shown in Figure 4 (see online Materials and Methods). The responses to 200  $\mu M$   $H_2O_2$  and to 2.5 Gy  $\gamma$ -radiation clustered together, that is, among 12 stress agents, including 10 DNA-damaging agents,  $\gamma$ -radiation at 2.5 Gy elicited the transcriptional response that was the closest to that of  $H_2O_2$ . We concluded that these similar transcriptional responses reflect similar damages in the cells.

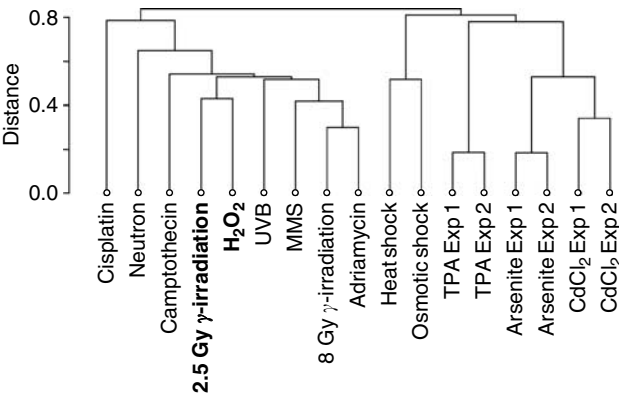
**Table 2** Error rates for supervised classification

|  | French error | CTB error | Global error |        |
|--|--------------|-----------|--------------|--------|
| (a) Classification based on all genes  |              |           |              |        |
| GPLS   | 17           | 7         | 12           |        |
| PAM  | 25           | 29        | 27           |        |
| RF   | 33           | 14        | 23           |        |
| LKSVM  | 25           | 7         | 15           |        |
| (b) Classification based on H <sub>2</sub> O <sub>2</sub> vs γ-radiation signature |              |           |              |        |
| GPLS   | 8            | 21        | 15           |        |
| PAM  | 25           | 29        | 27           |        |
| RF   | 42           | 7         | 23           |        |
| LKSVM  | 25           | 7         | 15           |        |
|  | French error | CTB error | Global error | P      |
| (c) Classification based on homologous radiation signature                         |              |           |              |        |
| GPLS   | 17           | 21        | 19           | 0.0038 |
| PAM  | 25           | 21        | 23           | <0.001 |
| RF   | 42           | 21        | 31           | 0.063  |
| LKSVM  | 8            | 21        | 15           | 0.0038 |

CTB=Chernobyl Tissue Bank; GPLS=generalised partial least-square; LKSVM=linear kernel support vector machines; PAM=prediction analysis of microarray; RF=random forest. Classification and validation procedures are described in Materials and Methods.



**Figure 3** Top 256 most classifying genes according to GPLS/*t*-statistics. Chernobyl Tissue Bank samples are in red and French samples in black in the top colour bar. Data are ordered with two-way hierarchical clustering for the sake of display clarity.



**Figure 4** Hierarchical clustering of transcriptional responses of the B-lymphocyte TK6 cell line to various stress agents. Expression data are from Amundson *et al* (2005). The responses to 200  $\mu$ M of H<sub>2</sub>O<sub>2</sub> and 2.5 Gy  $\gamma$ -radiation cluster together. Abbreviations: MMS, methyl methane-sulphonate; TPA, 12-O-tetradecanoylphorbol 13-acetate; UVB, ultraviolet (280–320 nm). The suffixes 'Exp1' and 'Exp2' stand for replicated experiments.

**Chernobyl Tissue Bank and French tumours are accurately classified on the basis of genes regulated differently in  $\gamma$ -radiation and H<sub>2</sub>O<sub>2</sub> responses**

The transcriptional responses to  $\gamma$ -radiation and H<sub>2</sub>O<sub>2</sub> are broadly similar; however, some genes are expressed differently between the two *in vitro* assays. We reasoned that these expression differences may mirror subtle underlying  $\gamma$ -radiation and H<sub>2</sub>O<sub>2</sub> susceptibility differences between CTB and French tumours that could be used for classification.

We found 293 genes in the 1451 published by Amundson *et al* (2005) with a fold change greater than 1.5 between the  $\gamma$ -radiation (2.5 Gy) and the H<sub>2</sub>O<sub>2</sub> responses (200  $\mu$ M). These responses were measured in B lymphocytes, whereas our goal was to classify thyroid tumours. Thus, we removed immune system-specific genes from the set of 293 genes (see online Materials and Methods). This filtering left 162 genes. Among them, 118 were spotted on our microarrays. They are listed in Supplementary Table S2 and will be referred to thereafter as the  $\gamma$ -radiation *vs* H<sub>2</sub>O<sub>2</sub> signature. Note that it was derived independently of our PTC data.

Next, we applied the same four classification algorithms as above except that only the independently selected 118 genes were used. Error rates (Table 2B) were comparable to those obtained in Table 2A, where the classifying genes were selected from a list of 8000. Again, all four algorithms classified the tumours with an error rate  $\leq 27\%$ , GPLS/*t*-test and LKSVM/RFE being the most accurate with an error rate of 15%. This result shows a relation between the  $\gamma$ -radiation *vs* H<sub>2</sub>O<sub>2</sub> signature and CTB and sporadic carcinomas distinction, which could reflect the underlying aetiology of CTB and French tumours.

**Chernobyl Tissue Bank and French tumours are accurately classified on the basis of 13 genes involved in homologous recombination**

To focus better on which elements of the DNA-damage response may differ between CTB and French tumours, we investigated if genes involved in the different DNA repair mechanisms led to accurate classification. We collected from the Human DNA Repair Genes database (Wood *et al*, 2001, 2005), all the genes known to be involved in base-excision repair, mismatch-excision repair, nucleotide-excision repair, homologous recombination and non-homologous end joining. The signature from each one of these five repair mechanisms was then used to classify the CTB and French

**Table 3** Homologous recombination gene signature

| Symbol  | Name  |
|---------|---|
| XRCC2   | X-ray repair complementing defective repair in Chinese hamster cells 2              |
| SHFM1   | Split hand/foot malformation (ectrodactyly) type 1                                  |
| RAD51C  | RAD51 homologue C ( <i>Saccharomyces cerevisiae</i> )                               |
| MUS81   | MUS81 endonuclease  |
| RAD51LI | RAD51-like 1 ( <i>S. cerevisiae</i> )   |
| RAD51   | RAD51 homologue (RecA homologue, <i>Escherichia coli</i> ) ( <i>S. cerevisiae</i> ) |
| RAD50   | RAD50 homologue ( <i>S. cerevisiae</i> )  |
| RAD54B  | RAD54B homologue  |
| RAD54L  | RAD54-like ( <i>S. cerevisiae</i> )   |
| NBS1    | Nijmegen breakage syndrome 1 (nibrin)   |
| RAD52   | RAD52 homologue ( <i>S. cerevisiae</i> )  |
| XRCC3   | X-ray repair complementing defective repair in Chinese hamster cells 3              |
| BRCA1   | Breast cancer 1, early onset  |

Only homologous recombination genes represented on our microarrays are listed (see main text).

tumours. These signatures contain few genes and were compiled from a source curated by DNA repair experts. Therefore, we skipped the gene selection step, which in turn alleviates the need for time-consuming internal cross-validation. The resulting computational gain made it tractable to run an additional statistical control: all five classification tasks were rerun 1000 times with the CTB and French labels randomly assigned to the tumours to estimate *P*-values, that is, the odds that the classification error was as low as the one observed with the actual data. Besides this, the classification proceeded exactly as above.

The classification error rates were high for base-excision repair, mismatch-excision repair and nonhomologous end joining, regardless of the algorithm (not shown). The nucleotide-excision repair signature produced an error rate of 27% with RF, but  $\sim 50\%$  with GPLS, PAM and LKSVM. In contrast, the homologous recombination signature (Table 3) led to a classification below 31% for all four procedures, below 20% for two and equal to 15% for LKSVM (Table 2C).

The *P*-value for RF, 0.064, was slightly above the 0.05 significance standard. All the other *P*-values were highly significant and remained below 0.02 after adjusting for the fact that five classification tasks were being examined (using Bonferonni correction, i.e., multiplying the *P*-values by 5). This suggests that homologous recombination, which repairs double-strand breaks, operates differently in CTB and French tumours or in the associated normal tissues. None of the homologous recombination signature genes are part of the 118 genes of the  $\gamma$ -radiation *vs* H<sub>2</sub>O<sub>2</sub> signature. Thus, the homologous recombination and  $\gamma$ -radiation *vs* H<sub>2</sub>O<sub>2</sub> signatures are nonoverlapping. They are thus two different signatures supporting a link between radiation and the CTB/French PTC expression differences.

**DISCUSSION**

We compared French and CTB tumours at the level of their global expression profiles, that is, of their overall phenotype. Hierarchical clustering and multidimensional scaling failed to uncover a large-scale difference between them. Note that, would such difference exist, our preliminary study (Detours *et al*, 2005) would have revealed it. Thus, the conclusion of pathologists that sporadic and radiation-induced PTCs are the same type of lesions is supported by expression data.

The similarity of expression profiles on a global scale, as observed with hierarchical clustering performed on all genes, does

not preclude that small groups of genes differ between these profiles. Supervised classification is the tool of choice to evaluate whether a group of genes can be exploited to discriminate different classes of tumours (Allison *et al*, 2006). Four linear classification algorithms assigned the tumours to the French or CTB groups with an error ranging from 12 to 27%, and  $\leq 15\%$  for two algorithms. These figures are typical of properly designed microarray studies (Ntzani and Ioannidis, 2003), and compare very favourably with histopathological diagnosis accuracy in the field of thyroid tumours (Baloch *et al*, 2001; Hegedus, 2004; Clary *et al*, 2005). The stability of the gene lists uncovered through supervised classification is problematic, even in studies using hundreds of samples (Ein-Dor *et al*, 2005; Michiels *et al*, 2005). Clearly, much larger studies will be needed to list exactly and exhaustively the discriminating genes, and validate them over a larger group. Nevertheless, our results strongly suggest that such genes exist: accurate classification of CTB and French tumours is possible on the basis of their expression profiles.

Initial reports of a low BRAF mutation frequencies in post-Chernobyl tumours (Nikiforova *et al*, 2004) and of a large impact of BRAF on gene expression (Giordano *et al*, 2005) raised the possibility of a radiation damage signature based on the mutational status of the tumours. The frequency of BRAF V600E mutation was similar, 38%, in our French and CTB tumours. Our analysis does not exclude the possibility of other damage signatures yet to be identified.

Radiation is a proven causing factor for PTC and a number of arguments support the view that H<sub>2</sub>O<sub>2</sub>-induced damage also contributes to initiate these tumours (see Introduction). Taking advantage of published data on the transcriptional responses of human lymphocytes to 13 stress agents (Amundson *et al*, 2005), we investigated how similar the responses to H<sub>2</sub>O<sub>2</sub> and  $\gamma$ -radiation are. We found that among 10 genotoxic agents, H<sub>2</sub>O<sub>2</sub> at 200  $\mu$ M elicits the response most similar to that of radiation at 2.5 Gy. This strengthens the argument for H<sub>2</sub>O<sub>2</sub> as a PTC-causing agent, as this similarity most probably mirrors a similarity of the damage inflicted by H<sub>2</sub>O<sub>2</sub> and radiation. This similarity is in line with the finding that French and CTB tumours have similar global profiles. Interestingly, Xiong *et al* (2005) demonstrated that the number of chromatid breaks per cell following  $\gamma$ -irradiation was significantly higher in the lymphocytes of 57 PTC patients with no documented exposure to radiation than in the lymphocytes of healthy controls. This difference could be related to impaired homologous recombination as the 18067T allele variant of XRCC3 was more frequent in 134 thyroid cancer patients than in 166 healthy patients in another study (Sturgis *et al*, 2005).

Transcriptional responses to H<sub>2</sub>O<sub>2</sub> and  $\gamma$ -radiation are similar relatively to other responses to genotoxic agents. However, 118 genes regulated differently in response to H<sub>2</sub>O<sub>2</sub> and radiation were uncovered and could be used to classify CTB and French tumours with an error as low as 15%. This is straightforward evidence that at least some of the genes associated with these tumours are also associated with the response to their presumed respective aetiological agent.

Next, we investigated whether French and CTB tumours could be classified on the basis of five signatures covering the genes involved in the five major DNA repair mechanisms: base-excision repair, mismatch-excision repair, nucleotide-excision repair, homologous recombination and nonhomologous end joining (Wood *et al*, 2001, 2005). The homologous recombination signature, which shares no genes with the H<sub>2</sub>O<sub>2</sub> vs  $\gamma$ -radiation signature, led to classification errors ranging from 15 to 31%. None of the other four signatures led to accurate classification. The specificity for the homologous recombination effect, and the good classification of CTB and French tumours using the 118 genes regulated differently in response to H<sub>2</sub>O<sub>2</sub> and radiation, make unlikely the confounding effect of age- or ethnicity-related factors. The fact that homologous recombination is involved in double-

strand break repair fits the notion that radiation causes more double-strand breaks than H<sub>2</sub>O<sub>2</sub>. Nevertheless, although potential confounders are controlled for by the use of patient-matched adjacent tissues, they are not formally ruled out in our study. This will become possible in the future as tumours from younger Ukrainian patients born after 1987 become available.

Thus, several independent gene-expression signatures separate our CTB and sporadic PTCs. These subtle expression differences between CTB and French tumours must be interpreted in light of the fact that the tumours investigated were removed > 15 years after the Chernobyl accident. Thus, any discriminating gene-expression signature had to be sustained over this time interval. DNA damage resulting from radiation, however, is typically mostly repaired within a time scale of hours. Consequently, either the reported signatures are 'damage signatures', that is, they are late results, from radiation-induced DNA damage (e.g. non- or incorrectly repaired damage), and/or they are 'susceptibility signatures', that is, they mirror radiation susceptibility factors pre-existing to the accident. The fact that one of the signature relies on the relative response to the two postulated causing agents ( $\gamma$ -rays and H<sub>2</sub>O<sub>2</sub>), and that the other relies on double-strand break repair genes, suggests that these signatures are related to the tumour-initiating mechanisms. This and the longlasting presence of these signatures support the susceptibility signature model. The recent finding that different TP53 alleles are associated with radiation exposure in adult PTC from Russian-Ukrainian patients (Rogounovitch *et al*, 2006) also supports this view. The susceptibility model, and the corollary that radiation susceptibility varies among individuals, may partly explain why only a minority of the population most exposed to radiation in Ukraine and Belarus developed PTC.

Thus, we interpret our findings as evidence for different and detectable cancer susceptibility factors underlying CTB and French tumours, which leads to several testable predictions. Expression ratios of tumours with respect to patient-matched adjacent tissues were measured. Hence, we could uncover susceptibility signatures only to the extent that they manifest themselves differently in the cancers and their adjacent tissues. We anticipate that the direct comparison of expression levels instead of expression ratios could lead to a stronger signature, possibly involving more genes. In addition, a radiation susceptibility signature could be present in healthy cells of any type in post-Chernobyl cancer patients. This, then, suggests the possibility of developing an expression-based *in vitro* test for radiation susceptibility. Finally, large-scale studies could uncover the genetic or epigenetic variations underlying the phenotypic differences reported in this paper. These concepts and approaches may apply to other types of cancers.

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